INVESTIGATIONS ON THE SEED PROTEINS OF THE *GRAMINEÆ* AND *LEGUMINOSÆ*

INAUGURAL DISSERTATION

BY

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Investigations on the Seed Proteins of the *Gramineae* and *Leguminosae*

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The reserve proteins in different seeds have been the subject of different researches, because, as *Vickery* has pointed out, "the simple plant seed, the ultimate source of all foodstuffs, is one of the most important organisms with which mankind is concerned" (1). The seed proteins mainly serve as reserve proteins, and during germination they are broken down into peptides and amino acids which are then transported to the growing parts, where they become available for protein regeneration (2). Some seed proteins serve other purposes, however, *e. g.*, as enzymes which assist in the breakdown or synthesis of proteins, starch and other products.

The purification and separation of different seed proteins has been of interest both from the practical and theoretical point of view, and biochemists have been working on this problem for more than 200 years. *Beccari* studied gluten from wheat as early as 1747. For references, see *Osborne*'s monograph "The Vegetable Proteins" (3), where the early investigations in this field are reviewed. However, the methods used for the preparation of seed proteins during the 19th century denatured the proteins, a fact which is quite clear to us with our present knowledge of the nature of proteins. During the last few decades many new and powerful methods of protein analysis have been worked out, and the time has come for a reassessment of the seed proteins.

The fundamental work in the field of seed proteins was done by *Osborne*, who started in 1891 and during the following thirty years published more than a hundred papers on this subject. For references, see (3). Many results obtained by *Osborne* and his co-workers are still valid, the greater part of his outstanding work being concerned with the isolation of seed proteins. In his earlier experiments he worked on the hypothesis that seed proteins from closely related plants were identical (1). Later, in collaboration with *Wells,* he was able to show by immunological tests that there are few, if any, seed proteins which are identical (4, 5, 6, 7). It is, however, very difficult to draw any important conclusions from their work, because no criterion of the purity of their protein fractions is available.

The first investigations which are described in the present paper were suggested by Professor *The Svedberg* who had the idea that botanical systematical conclusions could possibly be drawn from comparative ultracentrifugal studies of the seed proteins. *Gralén* and *Svedberg* were the first to work along these lines with their investigations of the soluble reserve-carbohydrates in the *Liliifloeae* (8). A great deal of work had to be done on the individual seed proteins, however, before the suggested comparative analyses could be carried out. This led the author to the investigations described in this paper.

Are the seed proteins individual proteins?

Osborne showed that the seed globulins from different plants could be separated into a few simple fractions, each probably containing only one globulin. The different protein fractions had different compositions, and *Osborne* thought that the different globulins were homogeneous and that they consisted of individual proteins. The elementary composition (C, H, N, S) of legumin from seeds of *Pisum sativum*, *Ervum Lens* and *Vicia Faba* was shown to be the same, and similarly for vicilin from these plants, but in all cases the composition of legumin was different from that of vicilin. Legumin from these three species contained 0.40 % S and vicilin only 0.16 % S (9).

Other recent work in this field has not confirmed *Osborne*'s results that the seed globulins are individual proteins. *Johns* and *Jones* (10, 11) showed that the globulins in the peanut, *Arachis hypogaea*, could be separated into two fractions — arachin and conarachin — which two globulins differed in sulphur, basic nitrogen, and amino acid content (12, 13). The major component, arachin, was shown by *Irving*, *Fontaine* and *Warnes* to consist of at least two electrophoretically different components (14, 15). *P. Johnson* and his co-workers showed by ultracentrifugation and electrophoresis that arachin contains two main components which may be dissociated or associated, according to the ionic strength and pH value, *i. e.*, they belong to the same dissociating system (16, 17, 18). No chemical analysis has been performed to confirm these results, however. Glycinin, the main globulin from the soy-bean, has also been shown to be inhomogeneous by chemical analysis (19, 20).

All these modern works on seed globulins have raised the very important question of whether or not these substances are individual proteins (21, 22). *Vickery* (1) says, that "with the possible exception of the major storage protein, there is little or no assurance that the fractions represent other than complex mixtures". *Rich* (23) suggests that the whole wheat protein should be regarded as a single substance which can be split into arbitrary fractions.

Quensel's work on the seed globulins of barley, *Hordeum vulgare*, also indicated that the seed proteins can be fractionated, and that ultracentrifugation separates a few fractions (24). By using *Osborne*'s preparation methods, extraction of the seed material with salt solutions,

precipitation of the globulins with $(NH_4)_2SO_4$ and dialysis to the salt-free state, he isolated from barley seeds 4 globulins, called α, β, γ and δ. The δ-component was obtained in low concentration and always together with the α- and γ-components, which could not be separated with the methods used by *Quensel*. The relative concentration of the two components could be changed, however. The β-component was always contaminated with low-molecular, inhomogeneous proteins. No chemical analysis of the different fractions is referred to in his paper.

Quensel's results are very important, but he did not actually succeed in isolating the individual globulin components from barley seeds. His results provide no answer to the questions of whether these components belong to a dissociating system or not, and whether the globulins are differentiated in the seeds before the extraction.

I. Investigation of the seed globulins of the *Gramineae*

1. *The occurrence of the globulin components in the seeds of different plant species* (25)

Quensel investigated the seed globulins of barley only. In order to determine whether similar globulins could be isolated from seeds of other plants belonging to the same plant family, *Quensel*'s methods were used in the study of seed globulins of 9 species belonging to the *Gramineae*. The results are presented in Table 1 and Figure 1.

Table 1. Seed globulins of species of the *Gramineae*.

Species	Components				Sedimentation constants s_{20} in *Svedberg units*			
Hordeum vulgare	α	β	γ	δ	2.5	6.2	8.3	12.0
Secale cereale	α	—	γ	—	2.6	—	8.2	—
Triticum vulgare	α	—	γ	—	2.5	—	8.2	—
Avena sativa	α	—	γ	—	2.6	—	8.1	—
Zea Mays	α	—	γ	—	2.6	—	8.5	—
Panicum miliaceum	—	—	γ	—	—	—	8.5	—
Phleum pratense	—	—	γ	—	—	—	8.2	—
Festuca pratensis	—	—	γ	—	—	—	8.3	—
Festuca rubra	α	—	—	—	2.4	—	—	—

From Table 1 it can be seen that barley, *Hordeum vulgare*, is the only species of those investigated for which the β- and δ-components can be isolated. The γ-component is present in the seeds of 8 of the 9 species examined. The only exception, *Festuca rubra*, has very small seeds with small amounts of reserve proteins, so that it is impossible to say whether the γ-component is present in these seeds or not, because its concentration may be to low. The α-component is present in the seeds of 6 of the species investigated. It is significant that no new globulin components of any kind could by found. These experiments permit us to draw the conclusion

that in the *Gramineae* each of the closely related species investigated contains mainly two globulin components with the sedimentation constants 2.5 S and 8.3 S. It would be necessary to treat a large number of plants, and to carry out accurate amino acid determinations and immunological tests, in order to draw any more general conclusions concerning the identity of these globulins from different plants.

2. *The differentiation of the globulins in the seeds*

Since it was impossible for *Quensel* to separate the α- and γ-components completely with the methods he used, precipitation with $(NH_4)_2SO_4$, centrifugation and electrophoresis, it is clear that these two globulins, if they are individual proteins, are very similar in their physical-chemical properties. Thus a method independent of the physical-chemical properties of these globulins must be used for their separation. It seemed possible that the two components are differentiated in the seeds. A passage in *Osborne*'s "The Vegetable Proteins" (3) indicated that work along these lines might lead to a positive result: "in the case of wheat the embryos are separated by the commercial process of milling in a nearly pure condition, and a study of this product has shown that those proteins which are obtained only in small quantity from the entire seed are present in relatively large amounts in this embryo meal". The globulins were isolated from wheat embryo by the methods indicated by *Quensel* (24), and ultracentrifugation showed that the embryo globulins consisted of the pure γ-component; it was impossible to establish the existence of the α-component in this fraction. The bran fraction, containing the protein-rich aleurone grains, and the endosperm (flour fraction) were investigated with the same methods (26). The results are shown in Figure 1. The bran fraction contains the γ-component in relatively high concentrations, and the endosperm contains mainly the α-component.

Analogous experiments were carried out with barley seeds. It is, however, impossible to isolate the embryo fraction by the commercial milling process. Therefore the barley seeds were cut into two parts with a razor blade. One fraction mainly consisted of embryo. The other fraction was called the residual fraction. Thus both these fractions contain part of the bran, and it is very probable that the embryo fraction in this case contains a great deal of the endosperm. The globulins were isolated from these two fractions, and the results, which are given in Table 2, agree with those obtained for wheat.

Table 2. Distribution of globulins in wheat and barley seeds.

	Embryo fraction			Residual fraction		
	α	β	γ	α	β	γ
Wheat	—	—	+++	+++	—	+
Barley	+	—	+++	+++	+++	—

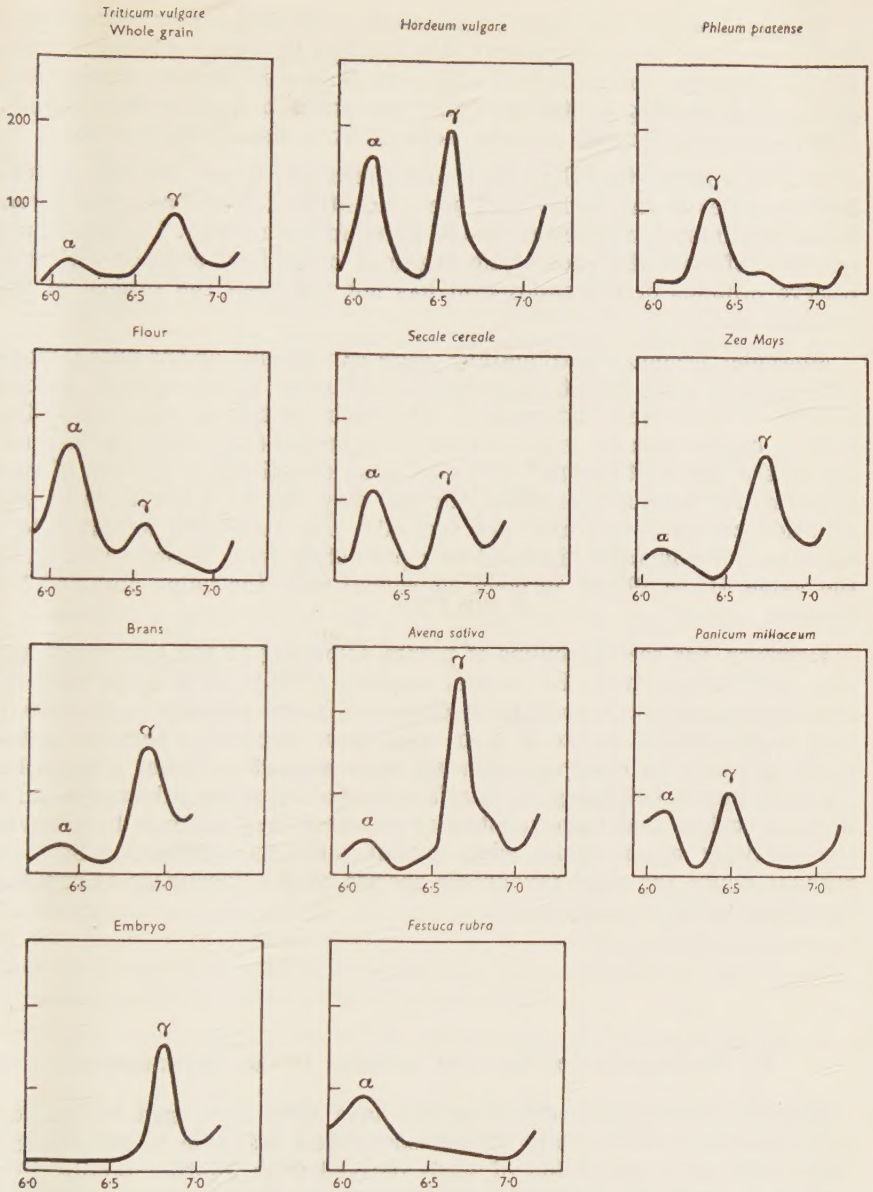

Fig. 1. Sedimentation diagrams of seed globulins from different species of the *Gramineae*. The four figures to the left refer to different fractions of seeds from wheat. In all the sedimentation diagrams in this paper the abscissae represent distance from the axis of rotation (cm), the ordinates represent the scale-line displacement (μ).

The residual fraction from barley seeds yielded the α-component perfectly free from the γ-component. The embryo fraction contained mainly the γ-component contaminated with a small amount of the α-component, probably depending on the fact that the embryo fraction was impure. The β-component could only be isolated from the residual fraction.

It is very interesting that *Engel* and *Heins* (27) found that the proteolytic enzymes in the barley seed are concentrated in the aleurone layer. Small amounts of enzyme can also be found in the embryo. The important question of how the proteolytic enzymes come into contact with the reserve proteins of the endosperm has not yet been the subject of investigation.

Molecular weight determinations were carried out on the pure γ-globulin isolated from wheat embryo. The diffusion constant and partial specific volume were determined. The value obtained, $M_\gamma = 210{,}000$, is 28 % higher than the value obtained by *Quensel* (22), probably depending on the fact that *Quensel* used a solution containing a mixture of two globulins for his determinations. In this way the determinations of the diffusion constants are not very accurate. The molecular weight determination for α-globulin, isolated in a pure state from barley seeds, gave the value $M_\alpha = 29{,}000$, in good agreement with the value obtained by *Quensel*.

However, the seed globulins of plants belonging to the *Gramineae* are very difficult to study, for several reasons. Firstly, they occur in very low concentrations. According to *Osborne* (3), the proteins in the wheat seed only contain up to 6 % of globulins. Secondly, they are relatively difficult to dissolve, even at ionic strengths higher than 0.25. Seeds of plants belonging to this family often contain substances dark brown in colour and these substances are often very difficult to remove, thus making observations with optical methods difficult. Wheat is without doubt the most favourable species of the *Gramineae* as regards investigation of seed globulins.

II. Investigation of the seed proteins of the *Leguminosae*

Since the analyses described so far have been concerned with seeds from one plant family only, it was considered desirable to supplement them with an investigation of seeds derived from another family. For certain reasons the family *Leguminosae* was chosen: for one thing the seeds of the *Leguminosae* contain large amounts of protein, and the protein metabolism has therefore often been the subject of investigation (1, 2). It is as well to point out one important difference between the *Gramineae* and the *Leguminosae:* the gramineous plants are monocotyledons and the leguminous plants are dicotyledons, which fact may be of importance as regards the storage of the reserve proteins in the seeds.

1. *Separation of the pea globulins* (25).

Osborne and his co-workers (28, 29) isolated from pea seeds three different protein fractions which they called vicilin, legumin and legumelin. Vicilin and legumin are globulins and legumelin is an albumin. During the first stage of their experiments they used 10 % NaCl extracts of the seed material. Vicilin, legumin and legumelin were precipitated from the extract by saturation with $(NH_4)_2SO_4$. By dialysis of the precipitate against water the proteins were dissolved, and when the salt concentration was lowered to a certain limit the two globulins precipitated, leaving the legumelin in the supernate. The globulins were dissolved in NaCl solution and separated by dilution in several stages, making use of the fact that the vicilin is more soluble at low salt concentration than is legumin (28).

Later, *Osborne* and *Harris* (29) separated vicilin from legumin by precipitation with $(NH_4)_2SO_4$ to 60 % saturation. According to them, legumin is precipitated and vicilin is not by this method. As a purity criterion of his pea globulin preparations *Osborne* used coagulation at 100° C, because vicilin is precipitated at this temperature but legumin is not.

Preliminary analyses of the pea globulins by the ultracentrifuge method clearly showed the existence of two globulin components. Different methods of separation were tried.

First, the method of *Osborne* and *Harris* (29) using fractional precipitation with $(NH_4)_2SO_4$ was tried. According to Fig. 2 some vicilin is precipitated at 63 % saturation together with legumin, and reprecipitation at 58 % saturation gives legumin preparations which are still contaminated with vicilin. Thus it is very probable that *Osborne* and *Harris*'s legumin preparations contained small amounts of vicilin. Their vicilin preparations were also probably contaminated with legumin, because Fig. 2 shows that all legumin has not been removed by precipitation at 63 % saturation. It is therefore inadvisable to draw important conclusions from their analyses and immunological tests because of the possible non-homogeneity of their protein preparations.

Fractionated precipitation of the globulins by dilution also gave unsatisfactory results.

After the discovery that the heavier of the two globulins, identical with *Osborne*'s legumin, could be precipitated isoelectrically in the pH range 4.5—5.5, in which range vicilin is soluble, this was used as the basis of an efficient method of separation. The sedimentation diagrams of the pure components are shown in Fig. 3. With this separation method the fractionation of vicilin and legumin could be carried out on a large scale, and it was possible to obtain several grams of the pure components.

Other experiments were carried out in order to decide whether or not these globulins are differentiated in the seeds in the same way as in the *Gramineae*. The answer was in the negative.

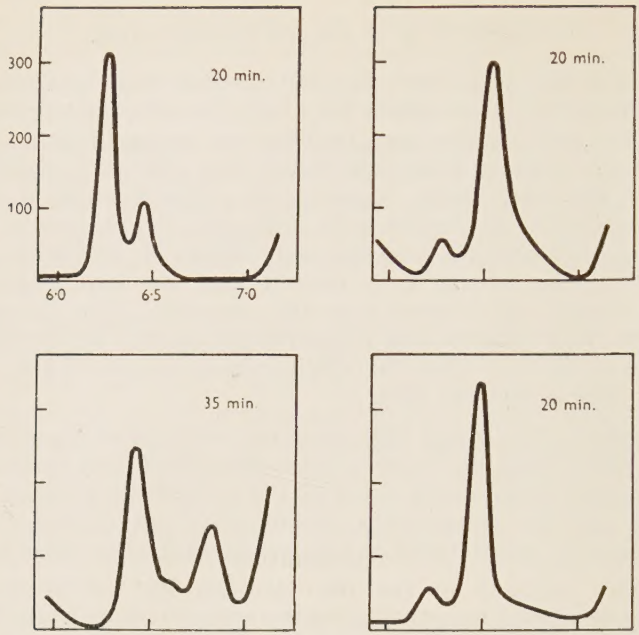

Fig. 2. Sedimentation diagrams of vicilin and legumin after fractional precipitation with $(NH_4)_2SO_4$.
Top left: initial solution.
Top right: precipitate at 63 % saturation.
Bottom left: precipitate between 63 % and 79 % saturation.
Bottom right: 63 % fraction reprecipitated at 58 % saturation.

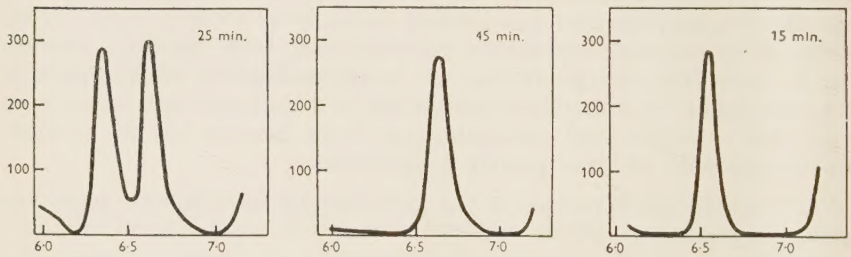

Fig. 3. Sedimentation diagrams of vicilin and legumin after separation by isoelectric precipitation.
Left: initial solution.
Centre: supernatant at pH 4.5 (vicilin).
Right: precipitate at pH 4.5 (legumin).

2. *Determinations of the molecular weights and electrophoretic analyses of pea globulins*

The molecular weights of the pure globulin components were determined (25). The diffusion experiments were carried out according to

Lamm's scale method. The molecular weight of vicilin was found to be $M = 186{,}000$. However, this value is perhaps not the true one because the sedimentation constant showed a clear tendency to increase at low concentrations. The value of s_{20} at zero concentration was taken as 8.10 S, this value having been obtained by extrapolation from a great number of determinations at low concentrations. This extrapolation is, however, not reliable. In the concentration range 0.3 - 1.5 % the unvarying value 7.4 S for the sedimentation constant was obtained, *i. e.*, about 10 % lower than the value used for the determination of the molecular weight of vicilin. This lower value of s_{20} gives $M = 170{,}000$ for vicilin. These determinations do at least give a good idea of the magnitude of the molecular weight of vicilin.

The molecular weight of legumin was determined as $M = 330{,}000$. In this case no dependence of s_{20} on concentration could be detected. *Sjögren* and *Svedberg* (30) obtained the value $M = 208{,}000$ for the molecular weight of legumin from vetch, *Vicia sativa*. This value is too low, because it is not corrected to water basis. If corrected, the value 300,000 is obtained. In view of their preparation methods it is also very probable that they used legumin preparations contaminated with vicilin. They thought that vicilin is not present in the seeds of vetch, in accordance with the findings of *Osborne* and *Campbell* (9), but the author has shown this to be incorrect.

The electrophoretic investigations on vicilin and legumin showed that these proteins are electrophoretically homogeneous (31), contrary to the findings of *Wetter* and *McCalla*, who detected at least three different components by electrophoresis of pea globulins (32). They mainly used *Osborne*'s precipitation methods for the isolation of the pea globulins, and their work indicated that *Osborne* was working with non-homogeneous vicilin and legumin preparations. But *McCalla* has gone too far when he says as a result of his electrophoretic studies of pea proteins: "Thus pea proteins, like most others studied, are mixtures of components, but these cannot be satisfactorily separated by usual methods of preparation" (22). The author has shown that this can be done by using a careful preparation technique.

The isoelectric point was found to be pH 5.5 for vicilin and pH 4.8 for legumin. The determinations for legumin were carried out at + 15° C, because of its insolubility at low temperature. The isoelectric precipitation of legumin in the pH interval 4—6 was studied by a nephelometric method. The maximum precipitation was found to occur at pH 5.0, *i. e.*, close to the isoelectric point of legumin, as determined electrophoretically.

The above results show that the pea globulins, vicilin and legumin, can be separated as homogeneous components when analyzed either by ultracentrifugation or in the electrophoresis apparatus. No changes of any kind have been observed when the globulins were dried at low temperature or their solutions stored for several months.

3. *Differences in amino acid composition of the pea proteins* (33)

Osborne and his co-workers carried out amino acid analyses of the pea proteins (34, 35, 36). Their results do not agree with those obtained by the author, however, doubtless because the old methods for amino acid determinations were not accurate enough, or their proteins were less well defined.

Determinations of tyrosine and tryptophan by measurement of absorption in the ultra violet region showed that the tyrosine content is about the same in the two globulins. The tryptophan content, however, is about three times higher in legumin than in vicilin. Clear differences could also be observed in the arginine, histidine and glutamic acid contents and in the distribution of acid, neutral and basic amino acids. A comparison of the amino acid composition of the pea globulins and other seed globulins investigated showed that the pea globulins are very rich in acid amino acids. This fact is not in agreement with the ideas of *Smith* and *Greene*, according to whom seed globulins are similar in composition (37, 38).

Only the pea globulins have been described so far. Several other proteins can be extracted from pea seeds. These proteins are of an albumin nature, and were called legumelin by *Osborne* (9), who thought they were a homogeneous protein. Such is not the case. The albumins are separated from the globulins by dialysis against water. The albumin fraction is inhomogeneous, and gives two components in the ultracentrifuge.

Amino acid determinations were carried out on the albumin fraction to ascertain whether its composition is similar to that of the pea globulins. It was found not to be so. Characteristic of the albumin fraction is its very high tryptophan and lysine contents and low arginine content. The considerable difference between the composition of the albumin fraction and that of the pea globulins was pointed out by *Osborne* and *Heyl* (36). The albumins seem to belong to another type of protein, as was shown by *Osborne* and *Mendel* with nutritional experiments (39). Albumin (legumelin) had better nutritive properties, probably owing to its high content of lysine and tryptophan.

4. *The seed globulins of different species of the Leguminosae*

In order to find out whether vicilin and legumin are present in the seeds of other species belonging to the *Leguminosae*, the seeds from 31 different species from this plant family were examined. The globulins were extracted with 0.2 M NaCl, pH 7, and separated from the albumins by precipitation with $(NH_4)_2SO_4$ at 70 % saturation. After further purification by dialysis against water, the globulins were examined in the ultracentrifuge. The results are given in Table 3. As can be seen from this table, one globulin with about the same sedimentation constant as vicilin was found in the seeds of all species investigated. One globulin

Table 3. Sedimentation constants (s_{20}) of the globulin components of various *Leguminosae*.

(Results in *Svedberg* units.)

Acacia alata	1.3	7.9	11.6	—
A. decipiens	—	8.0	12.7	—
A. Farnesiana	—	8.0	—	—
A. longifolia	2.7	7.6	—	18.0
A. penninervis	1.5	7.4	—	18.8
A. saligna	—	7.8	13.7	—
A. verticillata	—	7.8	—	—
Arachis hypogaea	2.0	8.4	13.1	—
Astragalus galegiformis	—	8.3	13.2	—
Cytisus Laburnum	—	8.1	14.0	—
C. supinus	1.8	8.0	13.4	—
Dolichos Lablab	—	7.3	11.7	—
Ervum Lens	—	7.3	13.2	—
Genista tinctoria	—	8.5	13.3	20.3
Glycine Soja	—	8.0	13.1	—
Lathyrus Clymenum	—	7.6	13.0	—
L. odoratus	—	7.6	12.0	—
L. sativus	—	7.5	13.0	—
L. silvestris	—	7.5	13.0	—
Lotus Tetragonolobus	—	8.3	13.1	—
Lupinus albus	—	8.2	12.3	—
L. angustifolius	—	8.2	13.1	—
L. luteus	—	8.3	11.5	—
L. polyphyllus	—	8.7	12.2	—
Medicago sativa	—	6.8	11.4	—
Phaseolus coccineus	4.3	7.4	12.2	—
P. nanus	—	6.6	10.1	—
P. vulgaris	4.9	7.3	11.0	—
Pisum sativum	—	8.1	12.6	—
Trifolium hybridum	—	7.7	12.9	—
T. pratense	—	7.7	11.2	—
T. repens	—	7.3	—	18.2
Vicia Faba	—	7.1	11.8	—
V. sativa	—	7.1	11.9	—

corresponding to legumin was found in most species, excepting some *Acacia* species and *Trifolium repens*, which seeds contain very little protein, however. Thus the results obtained indicate that the seeds of the *Leguminosae* species investigated contain two main seed globulins which, to judge by their molecular weights, are probably similar to vicilin and legumin from the seeds of the pea. In some cases, however, one component with sedimentation constant less than that of vicilin was observed. This component is probably albumin. In some cases a component with $s_{20} \sim$ 18—20 S was observed. It consists of associated legumin. When a solution of legumin is heated to 70—90° C legumin is associated to large molecules with $s_{20} \sim 19$ S. It has been found possible to transform legumin quantitatively to associated particles by this method. Prolonged dialysis can also cause association. These association reactions have not been investigated in detail, however.

Table 4. Sedimentation constants and molecular

Globulin	Species	Plant family	Sedimentation constant (s_{20} in *Svedberg* units)
Excelsin	*Bertholletia excelsa*	*Lecithydaceae*	13.3
Edestin	*Cannabis sativa*	*Cannabaceae*	12.8
Amandin	*Prunus Persica*	*Rosaceae*	12.5
Pomelin	*Citrus Aurantium*	*Rutaceae*	11.4
Cocosin	*Cocos nucifera*	*Palmae*	12.0
Hippocastanin	*Aesculus Hippocastanum*	*Sapindaceae*	12.4
Globulin	*Beta vulgaris*	*Chenopodiaceae*	13.9
»	*Sinapis alba*	*Cruciferae*	12.7
Legumin	*Pisum sativum*	*Leguminosae*	12.6
Vicilin	» »	»	8.1
Arachin	*Arachis hypogaea*	»	13.1
Conarachin	» »	»	8.4
Glycinin	*Glycine Soja*	»	13.1
Globulin	» »	»	8.0
Phaseolin	*Phaseolus vulgaris*	»	11.0
Conphaseolin	» »	»	7.3
Globulin α	*Hordeum vulgare*	*Gramineae*	2.5
» β	» »	»	6.2
» γ	» »	»	8.3
» δ	» »	»	12.0
Avenalin	*Avena sativa*	»	2.6
Globulin	» »	»	8.1
Globulin α	*Triticum vulgare*	»	2.5
» β	» »	»	8.2

[1] Calculated from the sedimentation constant on the assumption of spherical

5. *Comparisons between the seed globulins of the Gramineae and the Leguminosae*

The investigation of the seed globulins of the *Gramineae* showed that the seeds of most species examined contained two main globulin components. The same result was obtained with species belonging to the *Leguminosae*. It is quite certain, however, that the seed globulins from the two plant families are not identical in their sedimentation behaviour. The conclusion drawn from these investigations is that species belonging to the same plant family probably have seed globulins of about the same molecular weights.

It is a striking fact that most seed globulins isolated so far have molecular weights of about 300,000 except those from the *Gramineae*. Table 4 shows the molecular weights of some seed globulins. From the results obtained in this field, it may appear as if the seed globulins from

weights of different seed globulins.

Diffusion constant D_{20} in units of 10^{-7} c. g. s.	Molecular weight	
4.26	295,000	*Meyer, K. H.*, Natural and synthetic high polymers. N. Y. 1950.
3.18	381,000	— » —
3.45	346,000	— » —
—	~ 300,000[1]	*Svedberg, T.* and *Pedersen, K. O.*, The ultracentrifuge, Oxford 1940.
—	~ 300,000[1]	— » —
	—	*Säverborn, S.* and *Danielsson, C. E.*, Svensk Kem. Tidskr. *55* (1943) 155.
5.3	250,000	*Danielsson, C. E.* and *Ingelman, B.*, Svensk Kem. Tidskr. *59* (1947) 162.
	—	*Danielsson, C. E.*, Unpublished.
3.49	330,000	*Danielsson, C. E.*, Biochem. J. *44* (1949) 387.
4.26	186,000	— » —
	—	— » —
	—	— » —
	—	— » —
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	—	— » —
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7.4	29,000	— » —
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3.6	210,000	— » —

particles.

most families are similar and are built up in accordance with simple rules. This is, however, probably not the case. Two important questions arise automatically. First, why are the molecular weights of the seed globulins so high? One possibility is that the protein molecules must have a minimum size in order not to diffuse within the seed. The second question is: Why have so many plants seed globulins of two different kinds? If the seed globulins only serve as reserve protein, *i. e.*, store a certain amount of nitrogen in the seed during its dormant period, this could be done with only one reserve protein. But all seeds investigated by the author contained two seed globulins which were different in molecular weight and probably in chemical composition. Now, if we assume that the reserve proteins store nitrogen not only in the amount required for the protein synthesis in the new plant, but also in a suitable form for this synthesis, it can be understood why there is

more than one reserve protein in the seeds. The reserve proteins are broken down during germination to amino acids and simple peptides, and these substances are used for the protein regeneration. If many different amino acids or polypeptides are stored as such in the reserve proteins, it is probable that all combinations of these substances cannot be incorporated in one simple protein molecule, and two or more reserve proteins are therefore required in the seeds. For a further study of these problems it would be necessary to use methods for analysing the sequence of amino acids in polypeptides. These questions are probably very difficult to solve with the methods available at present.

6. *The breakdown of the reserve proteins during germination* (40)

Protein breakdown in seeds during germination has been studied intensively. For references into this field, see (2). *Hartig* (41) was the first to observe that the reserve proteins are broken down during germination in order to give soluble nourishment to the growing parts. The investigations of *v. Gorup-Besanez* indicated that a proteolytic enzyme in the vetch seed breaks down the reserve proteins to amino acids (42, 43, 44, 45). But most of the work done in this field dealt with the dependence of the protein breakdown on carbohydrate concentration, which problem was solved by *Peach* (46). Very few references can be found to determinations of protein concentration in seeds at different stages of germination. In most cases only a very few stages have been investigated, and the methods used give little or no information as to the nature of the proteins studied; *e. g.*, *Prianischnikow* (47) determined "protein" concentration in vetch seeds after 0, 10, 20, 30, and 40 days' germination.

Thus the breakdown of individual reserve proteins has not been studied with modern methods. To illustrate how little knowledge we have in this field, I may quote *Lugg* (21), who wrote as recently as in 1949: "When a seed is germinated the transfer of nitrogen to the growing parts involves the breakdown (at least in part) of the seed protein. This is clear not only from analytical evidence represented by (a) the difference in composition between seed and seedling protein and (b) the fact that asparagine and glutamine often appear in large quantities, but from cytological considerations, namely, the improbability that protein, as such, migrates across cell boundaries."

In order to obtain some information in this field, germination experiments were carried out on pea seeds and the amounts of different proteins were determined at different stages of germination. The finely ground plant material was extracted with 0.2 M NaCl, pH 7. This solvent extracts low-molecular nitrogenous substances, albumins, vicilin and legumin. About 70—80 % of the total nitrogen of the plant material could be extracted in this way. By dialysis of the extract against the extraction buffer the low-molecular nitrogenous substances were removed. The two globulins were separated from the albumin fraction by dialysis against water. The different fractions could be determined quantitatively by nitrogen

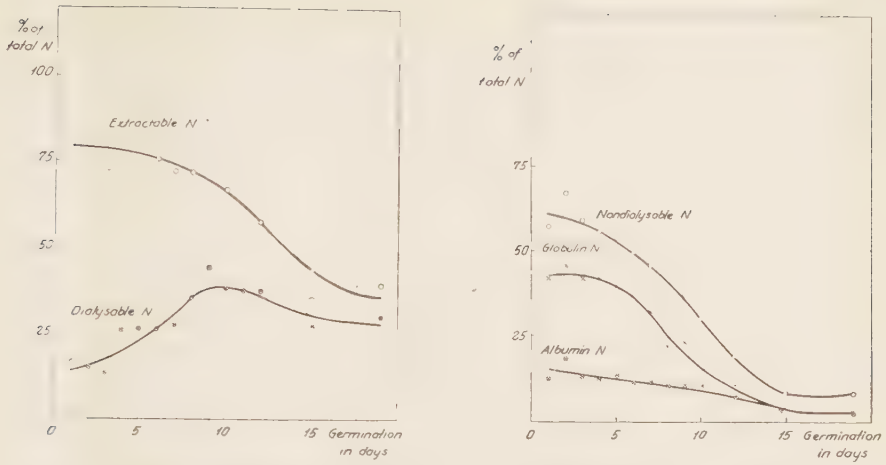

Fig. 4. Nitrogen distribution in pea seeds at different stages of germination.

determinations. The ratio of the amounts of vicilin and legumin was determined by ultracentrifugation. The results are shown in Figure 4.

The globulins, vicilin and legumin, are broken down very slowly during the first stage of germination. After about five days' germination the rate of breakdown increases, and after fifteen days about 90 % of the total amount of globulin has been broken down. The globulin breakdown takes place in time for the appearance of the new plant, that is to say, a pronounced maximum in the rate of breakdown can be seen when the new plant begins to form leaves. These experiments show that the globulins are reserve proteins, and their breakdown products are used for the synthesis processes in the new plant. A very important result is that both vicilin and legumin are broken down at the same rate, as was shown by ultracentrifugation. The concentration of vicilin was about 50 % higher than that of legumin during the whole germination process. It is impossible from these experiments to decide whether the two globulins serve different purposes during the germination.

The breakdown of the albumins is slow and occurs at a constant rate, and independently of the globulins. The albumins probably serve other purposes than the globulins during the germination, as is further evidenced by the fact that a proteolytic enzyme has been isolated from this fraction. This is yet another proof of the definite differences between globulins and albumins.

During the first ten days of germination, the amount of low-molecular nitrogenous substances increases owing to the rapid breakdown of proteins. After ten days, however, the amount of low-molecular nitrogenous substances decreases, probably depending on the fact that these substances are used for protein regeneration in the green leaves, as it may well be that this synthesis consumes low-molecular nitrogenous substances faster than they are supplied by decomposition of the reserve proteins.

These experiments are very simple, but they do give some information about the protein breakdown in seeds during germination. However, much work remains to be done in this field. One of the most important questions to be answered concerns the dependence of the breakdown of the individual proteins on carbohydrate concentration. The author has done no more than to suggest a suitable experimental method for a study of this kind.

7. *The synthesis of the reserve proteins during the ripening process* (48)

Emmerling found from his investigations on ripening seeds of the broad bean, *Vicia faba major*, that nitrogenous products are transported from different parts of the plant to the seed pods where they are stored before they are transported to the ripening seeds. (49, 50, 51). His results were confirmed by *Wassilieff* (52) and *Pfenninger* (53). *Schulze* and *Winterstein* (54) found different proportions of asparagine and arginine in the pods and the unripe seeds, indicating that the asparagine nitrogen in the pods is transformed to protein nitrogen in the seeds. Very little work has been done in this field using modern methods of protein and amino acid analysis, however. The author studied the synthesis of the well-defined reserve proteins in unripe pea seeds by the methods used in the investigations on the germination process. Fig. 5 shows that the amounts of protein N (nondialysable N) and globulin N (expressed as % of total N) increase mainly during the first stage of the ripening process. Albumin N, however, increases slowly with constant speed during the whole period of ripening. Low-molecular N decreases at the same rate as protein N increases. The curves obtained are very similar to those obtained from the germination experiments.

Ultracentrifuge experiments showed that vicilin is synthesized more rapidly than legumin during the first stages of ripening, while during

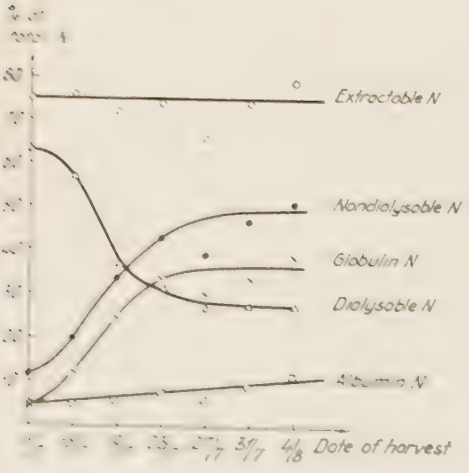

Fig. 5. Nitrogen distribution in pea seeds atdifferent stages of ripening.

the latter stages legumin formation dominates. The same result was obtained when seeds of the garden pea were analyzed. The well-defined globulin components are synthesized rather rapidly.

One of the most important questions with regard to protein synthesis is whether high-molecular intermediate products can be observed. The following experiments were carried out in order to get some information about such intermediate products.

Seeds at eight different stages of ripening were gathered and some were preserved by freezing to — 20° C and others were stored at room temperature. Extractions were carried out on the two lots obtained for each stage of ripening and the extracts were dialysed against the extraction buffer. In this manner the low-molecular nitrogenous compounds are removed from the extracts. Ultracentrifugal analyses of the non-dialysable nitrogenous products obtained by this method showed that ripening proceeds in detached, unripe seeds that are stored at room temperature, but in the seeds preserved by freezing the ripening process is arrested. During the first period of ripening, the seeds which had been kept at room temperature contained well-defined globulin components. No such components could be isolated from the seeds preserved by freezing, but these seeds contained water-soluble high-molecular nitrogenous substances, indicating the formation of high-molecular intermediate products during the first stage of the globulin synthesis. This idea was strengthened by determinations of the amino nitrogen content in water extracts from seeds preserved by freezing and seeds stored at room temperature. Very small differences in amino nitrogen content were obtained.

The author believes that detached, unripe seeds are excellent material for further studies of protein synthesis, especially if they are treated as described above, *i. e.*, if some seeds in the first stages of ripening are preserved by freezing and some are dried. It is then possible to study exactly the same seed material at two different stages of ripening. By determination of the amounts of protein, amino acids, amides, carbohydrates etc., the answers to some very important questions might be found, for instance, whether the nitrogen required for protein synthesis comes from the amino acids. Perhaps some enzyme systems (proteinase, trans-aminase) could be inactivated before the seeds are dried in order to study their role in protein synthesis.

8. *The proteolytic enzymes of seeds* (55)

As early as 1874, *v. Gorup-Besanez* studied the decomposition of the reserve proteins in seeds and reached the conclusion that it is caused by the proteolytic enzymes in the seeds (42, 43, 44, 45), and that this enzymatic decomposition gives amino acids. His hypothesis was accepted by *Schulze* and other important scientists in this field (2), and it laid the foundation of modern protein chemistry. It is a striking fact, however, that very little has been done in the field of proteolytic enzymes from seeds, especially from plants belonging to the *Leguminosae*. The

proteolytic enzymes have been studied in the case of cereals because of their importance in the baking and brewing processes. *Linderstrøm-Lang* and co-workers studied a proteinase from malt (56, 57) and *Mounfield* (58) and *Balls* and *Hale* (59) studied a proteolytic enzyme from wheat. The enzymes from wheat and malt have similar properties. The enzymes have probably not yet been obtained in a pure state, and much work remains to be done in this field. The enzymes from cereal seeds are easy to extract with water and the extracts have a rather high proteolytic activity. However, the proteolytic enzymes from leguminous seeds are much more difficult to study, owing to their low activities. There have been many attempts to isolate such enzymes, but very few of them have produced convincing results. The best results were obtained by *Davis* (60), who reported a proteinase from lima beans and by *Mergentime* and *Wiegand* (61) who studied the activity of pea proteinase at low temperatures. Very little is known of the absolute activities of these plant proteinases, however.

In the course of the germination experiments on pea seeds it could be seen that very large amounts of reserve proteins are broken down in a short time. The author extracted the proteolytic enzymes from pea seeds after 7—8 days' germination, that is at the stage of germination when the protein breakdown is most efficient. Glycerol extracts of the germinated pea seeds showed a weak proteolytic activity. By dialysing the glycerol extracts against water, it was possible to remove a great deal of the nitrogenous substances which were not enzymes, and the activity of the dialysed extract was about five times higher than that of the original extract, calculated on a nitrogen basis. After precipitation of the enzyme from the dialysed solution with $(NH_4)_2SO_4$, the activity obtained was twice as much as before precipitation.

Glycerol extracts were used in order to stabilize the proteinase. Later experiments, however, showed that the enzyme could be extracted with water, and somewhat higher activities were obtained by this latter method.

It is very difficult to remove all the non-enzyme protein substances which contaminate the proteinase. However, from the studies described above of the ripening process of pea seeds it can be seen, that during the first stage of ripening most of the nitrogenous substances in the seeds are low-molecular and can thus be removed from the extracts by dialysis. Thus, if unripe seeds contain a hydrolysing proteinase, the enzyme should be easier to purify from this material than from germinated seeds. Experiments showed these ideas to be correct and a strong hydrolysing proteinase could be isolated from ripening pea seeds by extraction with water. The enzyme was purified by dialysis and precipitation by $(NH_4)_2SO_4$.

The enzyme follows the albumin fraction, and its activity is not destroyed by drying *in vacuo*. The pH optimum of the pea proteinase was found to be pH 6.7 when splitting gelatin. The activity of pea proteinase is of the same order of magnitude as that of wheat proteinase,

and 1/100—1/200 of the activity of crystalline pepsin, when edestin is used as substrate.

Blagoveschenski (62) suggested that the proteolytic enzymes from plants are specific, *i. e.*, the seed globulins from one plant are broken down more rapidly by a proteolytic enzyme from the same plant then by other proteolytic enzymes. Such a specificity would certainly be remarkable. *Blagoveschenski*, however, used inhomogeneous globulin preparations as substrates, and his enzyme solutions were very crude because he used ground seedlings as enzyme preparations. Judging from his experimental procedure it appears that he was mainly determining the autolysis of the enzyme solution. Thus these results do not appear very convincing.

The author has isolated the proteolytic enzymes from seeds of wheat and pea seeds in a more active form than described earlier. Pure γ-globulin from wheat embryo and vicilin were used as substrates in these determinations. Digestion experiments using all four combinations of the two enzymes and two substrates showed no difference in specificity. The digestion was followed by formaldehyde titration and acetone titration in accordance with the *Linderstrøm-Lang* method (60). Both methods were used on a micro scale.

Thus *Blagoveschenski*'s ideas were shown to be wrong in this particular case when homogeneous and well-defined globulins were used as substrates and the enzymes were relatively pure.

Summary

1. The seed globulins of the *Gramineae* and the *Leguminosae* have been investigated.

2. Ultracentrifuge studies showed the existence of two globulin components in the seeds from both plant families.

3. In gramineous seeds the globulin components occur in different parts of the seeds. This is not true for leguminous seeds.

4. The globulins from gramineous seeds were obtained in a pure state by extracting different parts of the seeds.

5. The globulins, vicilin and legumin, from leguminous seeds were separated by isoelectric precipitation.

6. Vicilin and legumin were shown to be homogeneous when analyzed in the ultracentrifuge and in the electrophoresis apparatus.

7. The amino acid composition of vicilin and legumin is similar but not identical. Clear differences in the amino acid composition could be shown between the globulins and albumins from pea seeds.

8. Vicilin and legumin are not transformed into each other by dissociation or association.

9. The breakdown of the high-molecular pea proteins was studied during the germination process. The two globulins are broken down at the same rate and independently of the albumins.

10. During the ripening process vicilin and legumin are synthesized at different rates.

11. In detached, unripe peas the ripening process was arrested by freezing to −20° C. By this method high-molecular intermediate products from the synthesis of the globulins could be isolated.

12. The albumin fraction from pea seeds shows proteolytic activity. The enzyme was partly purified.

The present investigation has been carried out at the Institutes of Physical Chemistry and Biochemistry, University of Uppsala, Sweden.

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References

(1) *Vickery, H. B.*, Physiol. Revs. *25* (1945) 347.
(2) *Chibnall, A. C.*, Protein Metabolism in the Plant. Yale Univ. Press, New Haven, Conn. 1939, p. 86.
(3) *Osborne, T. B.*, The Vegetable Proteins, Longmans, Green, London 1924.
(4) *Wells, H. G.* and *Osborne, T. B.*, J. Infectious Diseases *8* (1911) 66.
(5) *Wells, H. G.* and *Osborne, T. B.*, J. Infectious Diseases *12* (1913) 341.
(6) *Wells, H. G.* and *Osborne, T. B.*, J. Infectious Diseases *14* (1914)377.
(7) *Lake, G. C.*, *Osborne, T. B.* and *Wells, H. G.*, J. Infectious Diseases *14* (1914) 364.
(8) *Gralén, N.* and *Svedberg, T.*, Biochem. J. *34* (1940) 234.
(9) *Osborne, T. B.* and *Campbell, G. F.*, J. Am. Chem. Soc. *20* (1898) 410.
(10) *Johns, C. O.* and *Jones, D. B.*, J. Biol. Chem. *28* (1916) 77.
(11) *Johns, C. O.* and *Jones, D. B.*, J. Biol. Chem. *30* (1917) 33.
(12) *Brown, W. L.*, J. Biol. Chem. *142* (1942) 299.
(13) *Brown*, W. L., J. Biol. Chem. *154* (1944) 57.
(14) *Irving, G. W.*, *Fontaine, T. D.* and *Warner, R. C.*, Arch. Biochem. *7* (1945) 475.
(15) *Fontaine, T. D.*, *Irving, G. W.*, and *Warner, R. C.*, Arch. Biochem. *8* (1945) 239.
(16) *Johnson, P.*, Trans. Faraday Soc. *42* (1946) 28.
(17) *Johnson, P.* and *Shooter, E. M.*, Biochim. et Biophys. Acta *5* (1950) 361.
(18) *Johnson, P.*, *Shooter, E. M.*, and *Rideal, E. K.*, Biochim. et Biophys. Acta *5* (1950) 376.
(19) *Smith, A. K.*, and *Circle, S. J.*, Ind. Eng. Chem. *30* (1938) 1414.
(20) *Csonka, F. A.*, and *Jones, D. B.*, J. Agr. Research *46* (1933) 51.
(21) *Lugg, J. W. H.*, Advances in Protein Chem. *5* (1949) 229.
(22) *McCalla, A. G.*, Ann. Rev. Biochem. *18* (1949) 615.
(23) *Rich, C. E.*, Cereal Chem. *13* (1936) 522.
(24) [illegible], O., Untersuchungen über die Gerstenglobuline. Dissertation, Uppsala 1942.
(25) *Danielsson, C. E.*, Biochem. J. *44* (1949) 387.
(26) *Säverborn, S.*, *Danielsson, C. E.*, and *Svedberg, T.*, Svensk Kem. Tid. *56* (1944) 75.
(27) *Engel, C.*, and *Heins, J.*, Biochim. et Biophys. Acta *1* (1947) 190.
(28) *Osborne, T. B.*, and *Campbell, G. F.*, J. Am. Chem. Soc. *20* (1898) 348.
(29) *Osborne, T. B.* and *Harris, I. F.*, J. Biol. Chem. *3* (1907) 213.
(30) *Sjögren, B.*, and *Svedberg, T.*, J. Am. Chem. Soc. *52* (1930) 3279.
(31) *Danielsson, C. E.*, Acta Chem. Scand. *4* (1950) 762.
(32) *Wetter, L. R.*, and *McCalla, A. G.*, Can. J. Research, *27* (1949) 96.
(33) *Danielsson, C. E.*, Acta Chem. Scand. *6* (1952) 139.
(34) *Osborne, T. B.*, and *Clapp, S. H.*, J. Biol. Chem. *3* (1907) 219.
(35) *Osborne, T. B.*, and *Heyl, F. W.*, J. Biol. Chem. *5* (1908) 187.
(36) *Osborne, T. B.*, and *Heyl, F. W.*, J. Biol. Chem. *5* (1908) 197.

(37) *Smith, E. L.*, and *Greene, R. D.*, J. Biol. Chem. *167* (1947) 833.
(38) *Bonner, J.*, Plant Biochemistry, Academic Press, New York 1950, p. 254.
(39) *Osborne, T. B.*, and *Mendel, L. B.*, Z. physiol. Chem. *80* (1912) 307.
(40) *Danielsson, C. E.*, Acta Chem. Scand. *5* (1951) 541.
(41) *Hartig, T.*, Entwicklungsgeschichte des Pflanzenkeims, Leipzig 1858 [Ref. from (2)].
(42) *Gorup-Besanez, E.*, Ber. *7* (1874) 146.
(43) *Gorup-Besanez, E.*, Ber. *7* (1874) 569.
(44) *Gorup-Besanez, E.*, Ber. *7* (1874) 1478.
(45) *Gorup-Besanez, E.*, Ber. *10* (1877) 780.
(46) *Peach, K.*, Planta *24* (1935) 78.
(47) *Prianischnikow, D. N.*, Landw. Vers. Sta. *46* (1895) 459.
(48) *Danielsson, C. E.*, Acta Chem. Scand. *6* (1952) 149.
(49) *Emmerling, A.*, Landw. Vers. Sta. *24* (1880) 113.
(50) *Emmerling, A.*, Landw. Vers. Sta. *34* (1887) 1.
(51) *Emmerling, A.*, Landw. Vers. Sta. *54* (1900) 215.
(52) *Wassilieff, N.*, Ber. deut. botan. Ges. *26* (1908) 454.
(53) *Pfenninger, U.*, Ber. deut. botan. Ges. *27* (1909) 227.
(54) *Schulze, E.*, and *Winterstein, E.*, Z. physiol. Chem. *65* (1910) 431.
(55) *Danielsson, C. E.*, Acta Chem. Scand. *5* (1951) 791.
(56) *Mill, C. K.*, and *Linderstrøm-Lang, K.*, Medd. Carlsberg Lab. *17* (1928) no. 10.
(57) *Linderstrøm-Lang, K.* and *Sato, M.*, Medd. Carlsberg Lab. *17* (1929) no. 17.
(58) *Mounfield, J. D.*, Biochem. J. *30* (1936) 549.
(59) *Balls, A. K.*, and *Hale, W. S.*, Cereal Chem. *15* (1938) 622.
(60) *Davis, W. R.*, Food Research *4* (1939) 613.
(61) *Mergentime, M.*, and *Wiegand, E. H.*, Fruit Products J. *26* (1946) 72.
(62) *Blagoveschenski, A.*, Biochem. J. *18* (1924) 795.
(63) *Linderstrøm-Lang, K.*, Medd. Carlsberg Lab. *17* (1927) no. 4.

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